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Gluconeogenesis in the Yolk Sac Membrane: Enzyme Activity, Gene Expression, and Metabolites During Layer Chicken Development

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Yolk sac membranes of layer eggs were collected daily (n = 7–9) from day three of incubation to day three post-hatch, and mRNA expression and activities were quantified for key gluconeogenesis enzymes (glucose-6-phosphatase, fructose-1,6-bisphosphatase, cytosolic and mitochondrial phosphoenolpyruvate carboxykinases, and pyruvate carboxylase). Lactate, triglycerides, non-esterified fatty acids, glycogen, and glucose in the yolk sac membrane, and blood glucose levels were also measured. The mRNA expression and activity were detected for all enzymes. Differences in expression levels and enzyme activities seemed to reflect the embryo's developmental environment and physiological demands at different developmental stages. During the first week to the mid-second week of incubation, the expression and activity of gluconeogenic enzymes and lactate concentrations were high, suggesting an active period of gluconeogenesis from lactate, reflecting possible hypoxia in the embryo before completed formation of the chorioallantoic capillaries. From the mid-second week to mid-third week, when embryos were in an aerobic state, the triglyceride and non-esterified fatty acid contents increased in the yolk sac. Triglycerides from yolk lipids are typically hydrolyzed to produce non-esterified fatty acids as an energy source, whereas the glycerol skeleton is used for gluconeogenesis. In the late third week, when embryos were considered to re-enter an anaerobic state, the mRNA expression and enzyme activity of only glucose-6-phosphatase were high and the amount of glycogen in the yolk sac was reduced. Therefore, it is suggested that gluconeogenesis activity is low during this period, and the carbohydrates stored in the yolk sac membrane are secreted into the blood as energy for hatching. This study confirmed the role of the yolk sac membrane as a vital gluconeogenic organ during chicken egg incubation.

Key words: chicken embryo, gluconeogenesis, lactate, PEPCK, yolk sac

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Introduction

In hatcheries, proper embryonic development requires provision of adequate temperature, humidity, and ventilation (with egg turning). Similar to the eggs of other avian species, almost all of the nutrients required for embryonic development are present in the yolk and albumen of chicken eggs. Respiratory gases (oxygen and carbon dioxide) and water vapor enter and exit the eggshell. However, unlike mammalian fetuses, which are connected to the maternal system through the placenta, no external nutrients are supplied to the developing chick embryo.

The ATP produced by the metabolism of nutrients present within the egg can serve as an energy source for embryonic morphogenesis. According to one estimate, a chicken egg with a wet weight of 60 g contains 88 kcal of energy; thus, hatching a 45 g chick corresponds to 64 kcal of energy (Etches, 1996) and the remaining energy is used for morphogenesis, including that released as heat. Moreover, eggs require reserves to cover the large amount of energy needed to crack the shell upon hatching, which can reach up to 10% of the energy needed for the entire developmental process depending on the bird species and shell thickness (Vleck and Bucher, 1998).

Hyperglycemia is a significant characteristic of all birds and is not found in other animal groups. Chickens do not have exceptionally high blood glucose levels compared to those of other birds, although they are still approximately twice as high as those of healthy humans (Martinez del Rio and Gutiérrez-Guerrero, 2020). Several studies have demonstrated that chick embryo blood glucose levels gradually increase during incubation (for

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example, Yarnell *et al*., 1966; Evans and Scholz, 1973; Lu *et al*., 2007), with Roy *et al*. (2013) reporting levels exceeding 100 mg/dL one-third into the incubation period and reaching 200 mg/dL at hatching. These facts indicate that the chick egg also requires sufficient storage of nutrients to cover the glucose needed for embryo development characterized by a hyperglycemia state. Therefore, in chicken and other bird embryos, the limited egg content (yolk and albumen nutrients) must be metabolized and available to the embryo for appropriate allocation to the different uses mentioned above.

Romanoff and Romanoff (1963) described the constituents of the chicken egg yolk and albumen: the yolk contains 49% water, 17% protein, 33% lipids, and 1% carbohydrates, and the albumen contains 88% water, 11% protein, and 1% carbohydrates. These values vary from egg to egg (Romanoff and Romanoff, 1963) depending on flock age (Fletcher *et al*., 1981; Rossi and Pompei, 1995) and breed (Hocking *et al*., 2003).

The yolk sac is a pouch-like structure that stretches over the yolk from approximately day two of incubation and completely envelops the yolk by approximately day nine (Romanoff, 1960a). The yolk sac membrane (or yolk sac tissue) that envelops the yolk (or yolk sac content) is the largest among the embryonic and extraembryonic organs that develop throughout the incubation period. The wet weight of the yolk sac membrane on day 17 of incubation (3.188 g, see Fig. 1) is approximately 6.7 times that of the embryonic liver (0.479 g) reported by Romanoff (1960b). The yolk sac membrane contains epithelia of endodermal origin (such as the liver and small intestine) that take up the yolk and

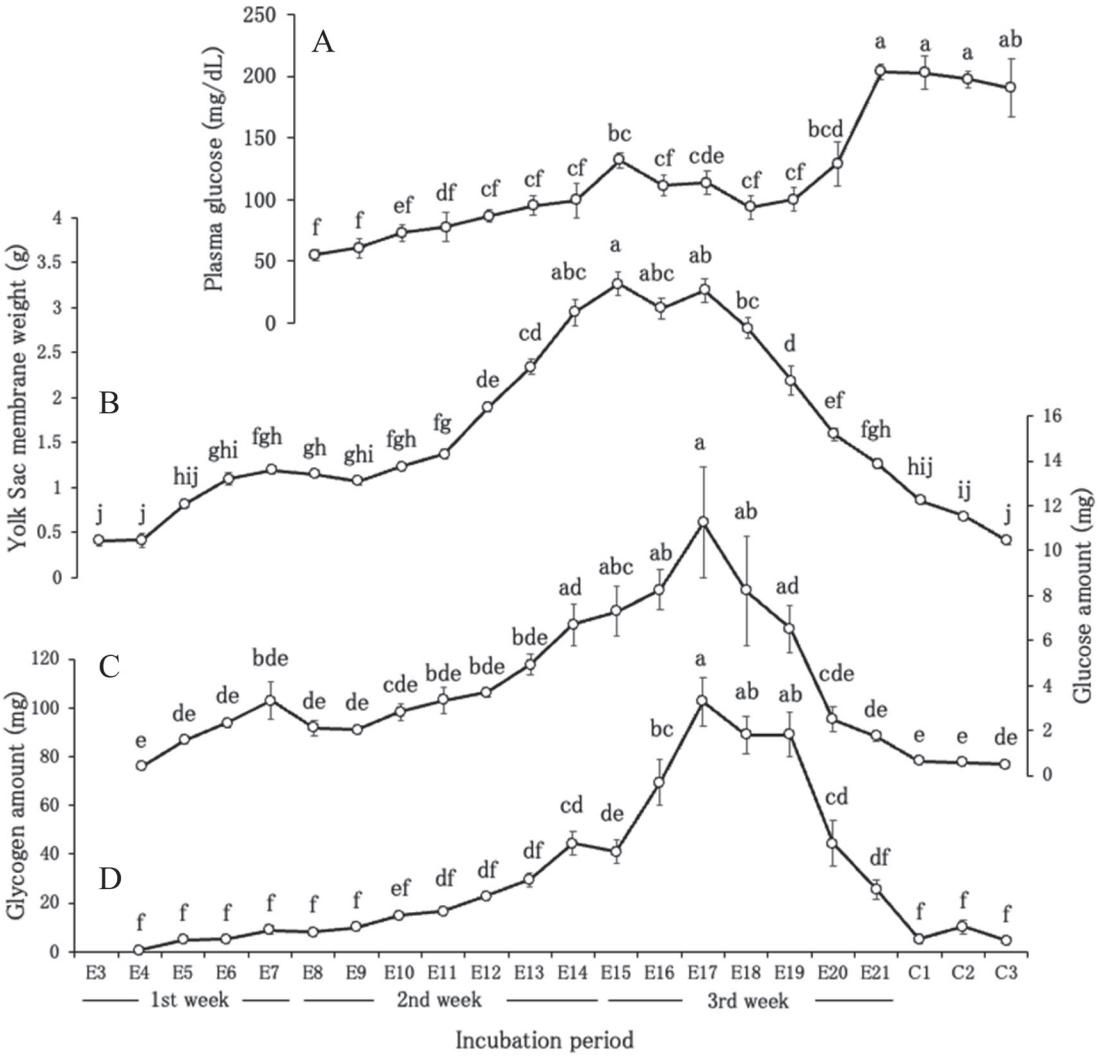

Fig. 1. **(A) Plasma glucose concentration, (B) wet weight of yolk sac membrane, (C) glucose amount, and (D) glycogen amount in the yolk sac membrane during embryonic development of the chicken (mean ± SEM, n = 7–9 per stage).** Means without a common letter are significantly different within the same measurement category ($p < 0.05$, Tukey–Kramer multiple-comparison test). "E" and "C" indicate the incubation day of the embryo and the day after hatching of the chick, respectively.

convert (or metabolize) the constituent nutrients into appropriate forms for use (Bauer *et al*., 2013). The metabolites produced during this process are delivered to the embryo via the yolk sac membrane vasculature. The egg white is transported to the embryo mostly via the yolk sac membrane (Yoshizaki *et al*., 2002).

Various metabolic enzymes have been identified in the yolk sac membrane (Nakazawa *et al*., 2011; Wong and Uni, 2021). In carbohydrate metabolism, the yolk sac membrane accumulates glycogen and expresses the enzymes responsible for its synthesis and breakdown (Willier, 1968; Yadgary and Uni, 2012). Enzymes involved in gluconeogenesis are also found in the yolk sac membrane (Nakazawa *et al*., 2011; Yadgary and Uni, 2012) where glucose is produced from non-carbohydrate sources. Gluconeogenesis may complement the low carbohydrate content among egg nutrients (approximately 1% as mentioned above).

Given that the yolk sac membrane consists of three different cell layers of endoderm, ectoderm, and mesoderm origin, Wong and Uni (2021) referred to this structure as more than simply a membrane but rather a crucial multifunctional organ during development. Therefore, clarifying the function and regulation of the yolk sac membrane is essential to understand the mechanisms that promote chick embryogenesis before the intestinal epithelium and liver become fully functional during development.

We previously reported that iodothyronine deiodinases (thyroid hormone-activating and -inactivating enzymes abundant in the liver of adult chickens) expressed in the yolk sac epithelium and showed dynamic levels during embryogenesis. Accordingly, we hypothesized that these enzymes are active in the yolk sac membrane, thereby regulating metabolism, including carbohydrate turnover (Too *et al*., 2017).

To further test this possibility, in this study, we focused on carbohydrate metabolism in the yolk sac membrane to clarify how this extraembryonic organ yields glucose from nutrients transferred from the yolk to meet the high metabolic demand during embryonic development. This is the first report to quantify the expression and activity of five key gluconeogenic enzymes [glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase (FBPase), phosphoenolpyruvate carboxykinase cytosolic isozyme (PEPCK-C), phosphoenolpyruvate carboxykinase mitochondrial isozyme (PEPCK-M), and pyruvate carboxylase (PC)] and related metabolites during the development of layer chickens every day from day three of incubation to day three post-hatch. Such a detailed understanding of the processes by which nutrients enclosed in the yolk by the mother hen are metabolized in the yolk sac membrane for embryonic development will provide fundamental knowledge for improving the productivity of poultry. Variation and availability (via the yolk sac membrane) of egg nutrient contents may affect embryonic development and the post-hatch growth, health, and welfare of chicks, not only in rapidly growing broilers but also in layers (Noble and Cocchi, 1990; van der Wagt *et al*., 2020).

Materials and Methods

Animals and sampling

Fertilized eggs from the layer strain Lohman Julia were purchased from Japan Layer, Gifu, Japan. The eggs were incubated at 37.8°C and 70% relative humidity. The day of incubation was labeled embryonic day 0 (E0). All embryos (chicks) were pipped with the beaks outside the eggshell at the E21 sampling period. The yolk sac membrane was sampled daily from E3 embryos to 3-day-old chicks (C3) (n = 7–9 per stage). The membrane was rinsed thoroughly with sterile phosphate-buffered saline (PBS) several times to remove remaining yolk, placed on a piece of filter paper to absorb excess PBS, weighed on an electronic balance, and then immediately frozen in liquid nitrogen and stored at –80°C until analysis. Blood was collected from the extraembryonic blood vessel at E8–E16, from the embryonic heart at E17–E20, and by quick decapitation at E21–C3 (n = 7–9 per stage). Plasma was purified from the blood and stored at –60°C. All animal experiments were approved by the Animal Research and Welfare Committee of Gifu University (No. 2021-229) and conducted in accordance with the principles and specific guidelines presented in Guide for the Care and Use of Agricultural Animals in Japan.

Quantification of plasma glucose

The plasma glucose concentration was measured using the glucose oxidase method with a Glucose CII Test kit (Wako Pure Chemical Industries, Osaka, Japan), as recommended by the manufacturer (n = 7–9).

Quantification of glucose and glycogen in the yolk sac membrane

A 100 mg sample of the yolk sac membrane was mixed with 500 μL of citrate buffer solution (0.5 M, pH 4.2), homogenized on ice using a micro-homogenizer, and transferred to a microcentrifuge tube. After heating at 100°C for 5 min, the sample was centrifuged (13,000 ×*g*, 5 min, 4°C) and the supernatant was collected. Glucose in the yolk sac membrane was measured using the Glucose CII Test kit (Wako), as recommended by the manufacturer (n = 7–9).

The glycogen content of the yolk sac membrane was measured according to the method described by Murat and Serfaty (1974). In brief, 40 μL of the yolk sac membrane sample used for glucose measurement or preparation of the glycogen standard solution was mixed with 100 μL (70 U) of amyloglycosidase (Sigma-Aldrich, St. Louis, MO, USA) enzyme solution (0.1 M citrate buffer, pH 4.2). After incubation at 30°C for 3 h, the reaction was stopped by heating at 100°C for 5 min. Glucose was quantified again, and the difference in glucose concentration before and after the amyloglycosidase enzyme reaction was considered to represent the conversion of glucose to glycogen. This amyloglycosidase reaction at 30°C for 3 h converted 99.7 ± 0.4% (mean ± standard error, n = 5) of the glycogen standard to glucose (data not shown). The total amount of glycogen in the yolk sac membrane was calculated by multiplying the glycogen concentration by the wet weight of the yolk sac membrane (n = 7–9).

Quantification of lactate in the yolk sac membrane

A 500 mg sample of the yolk sac membrane and 2.5 mL of perchloric acid (1 M) were added to a glass stirrer, completely mashed, and centrifuged at 3,000 ×*g* for 10 min. Subsequently, 0.02 mL of methyl orange indicator (concentration adjusted by adding 50 mg of methyl orange to 100 mL of distilled water) and potassium carbonate solution (5 M, 0.1 mL) were added and stirred. The potassium carbonate solution was further added until the color of the solution changed, the solution was then left on ice for 10 min, and the supernatant was collected. The lactate quantification assay was performed with 0.2 mL of the obtained extract using Detaminer LA (Minaris Medical, Tokyo, Japan), as recommended by the manufacturer (n = 7–9).

Quantification of non-esterified fatty acids and triglyceride in the yolk sac membrane

Total lipids were extracted from the yolk sac membrane using the method described by Folch *et al*. (1957). In brief, the yolk sac membrane (100 mg) was accurately weighed and 10 times its volume of distilled water (w/v) was added to obtain a concentration 0.1 mg/μL. After bead crushing (4,000 rpm, 1 min) of the tissue solution using a crushing device, 440 μL of the crushed solution was transferred to a new tube to which 1,100 μL of a methanol/chloroform solution (1:2) was added and mixed well. The mixture was centrifuged at 15,000 ×*g* for 5 min and 400 μL was collected from the lower organic layer. After heating and drying at 80°C for 15 min, 100 μL of methanol was added, and the solution was dispersed with sonication homogenizer and stored at –20°C. At the time of quantification, the solution was dispersed using a sonication homogenizer.

The concentration of non-esterified fatty acids (NEFAs) in the yolk sac membrane was measured using the LabAssay NEFA (FUJIFILM Wako Shibayagi, Gunma, Japan), based on the acyl-CoA synthetase (ACS)-acyl-CoA oxidase (ACOD) Method, as recommended by the manufacturer (n = 7–9). The concentration of triglycerides in the yolk sac membrane was measured using the LabAssay Triglyceride (Shibayagi), based on the glycerol-3-phosphate oxidase (GPO)-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS) Method, as recommended by the manufacturer (n = 7–9).

The results were calculated as the total amounts of NEFA and triglycerides in the yolk sac membrane by multiplying the respective NEFA and triglyceride concentrations by the wet weight of the yolk sac membrane.

Quantification of mRNA expression

Total RNA was extracted from frozen yolk sac membranes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNase-free DNase I (gDNA Remover, TOYOBO, Osaka, Japan) was used to eliminate genomic DNA according to the manufacturer's instructions. Total RNA (1 μg) was then reverse-transcribed into first-strand cDNA using ReverTra Ace (TOYOBO) and a random primer (TOYOBO).

mRNA levels were determined by real-time polymerase chain reaction (PCR) performed in an Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA) with a two-step standard cycling program for the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent). Each single reaction comprised 10 μL of qPCR Master Mix, 0.8 μL of each 10 μM forward and reverse primer, 7.4 μL of sterile water, and 1 μL of template cDNA. The cycling program consisted of 3 min of initial denaturation at 95°C, followed by 40 cycles of 5 sec of denaturation at 95°C and 20 sec of annealing and extension at 60°C. The amplification program was followed by dissociation curve analysis to detect non-specific amplification. Each experimental and standard sample was assayed in duplicate. Relative expression values were calculated according to the standard curve method using the software installed in the Mx3000P System with a serial dilution of pooled cDNAs as the standard. No-sample controls were included to confirm the specificity of reverse transcription and PCR amplification.

Using real-time PCR, *18S* ribosomal RNA and *GAPDH* were found to be the best housekeeping genes, because we confirmed that they did not show significant changes in the yolk sac membrane throughout development (Too *et al*., 2017). The mRNA expression levels were corrected for the mean values of these two housekeeping genes (n = 7–9).

The primers used for real-time PCR are listed in Table 1. The primers for *G6Pase, FBPase, PEPCK-C, PC, 18S* rRNA, and *GAPDH* have been previously reported for *Gallus*. The primers for *PEPCK-M* were designed from *G. gallus* genomic sequences in the GenBank database using the online primer design software Primer3 (Untergasser *et al*., 2012).

Conventional PCR for the glucose transporter *GLUT2* was performed using the chicken primer set reported by Rice *et al*. (2014) (F: GAAGGTGGAGGAGGCCAAA, R: TTTCATCGGGTCACAGTTTCC). The following PCR program was employed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA): initial denaturation for 2 min at 94°C followed by 30 cycles of 15 sec at 94°C, 30 sec at 60°C, and 1 min at 68°C using KOD -Plus- DNA polymerase (TOYOBO).

Quantification of enzyme activity

Preparation of the enzyme solution

The enzyme solution was prepared using a modification of the method described by Utter and Keech (1963). A 0.5 g tissue sample was accurately weighed and placed in 2.5 mL of a 0.25 M sucrose/1 mM EDTA-2Na solution, homogenized under ice-cold conditions, and centrifuged (600 ×*g*, 4°C, 15 min) to precipitate cell nuclei and cell membrane fractions. The supernatant was collected and further centrifuged (8,000 ×*g*, 4°C, 15 min) to precipitate the mitochondrial fraction, and a portion of the supernatant was used for G6Pase activity measurement. The supernatant was further centrifuged (105,000 ×*g*, 4°C, 60 min) and fractionated into soluble and microsomal fractions. The former was used to measure FBPase and PEPCK-C activities. The mitochondrial fraction was resuspended in 2 mL of 0.25 M sucrose/1 mM EDTA-2Na solution, centrifuged (8,000 ×*g*, 4°C, 10 min), and the mitochondrial fraction was precipitated once more. This solution was added to the precipitate and resuspended as the enzyme solution of the mitochondrial fraction, which was used to

Table 1. **Primers used for real-time PCR.**

Gene Name	Accession no.	Primer sequence (5′ → 3′)	Amplicon length (bp)	Reference
G6Pase	XM_42201.2	F: CATGTACTTCACTTACTTTCCTCC R: TATTGTATCAGCGTGGCGTA	137	Roy *et al*., 2013
FBPase	AJ276212	F: TTCCATTGGGACCATATTTGG R: ACCCGCTGCCACAAGATTAC	100	Yadgary and Uni, 2012
PEPCK-C	M14229.1	F: TGCTGGTGTGCCTCTTGTAT R: CACACGGGAATTCTCTCCAT	295	Roy *et al*., 2103
PEPCK-M	NM_205470.1	F: ACACCATCTTCACCAACGTG R: GTCCATAATGGGGCACTGAT	191	Present study
PC	AF509529.1	F: TGGGGCATAAATTCAAGGAG R: GAATTCGACCACGGAGAGAG	194	Roy *et al*., 2013
18s rRNA	AF173612.1	F: TCAACTTTCGATGGTACTGTCTGTG R: CTTGGATGTGGTAGCCGTTTCT	106	Roy *et al*., 2013
GAPDH	NM_204305	F: GAAGCTTACTGGAATGGCTTTCC R: GATATCATCATACTTGGCTGGTTTCTC	97	Van Herck *et al*., 2012

G6Pase: glucose-6-phosphatase; FBPase, fructose-1, 6-bisphosphatase; PEPCK-C: phosphoenolpyruvate carboxykinase cytosolic isozyme; PEPCK-M: phosphoenolpyruvate carboxykinase mitochondrial isozyme; PC: pyruvate carboxylase.

measure PC and PEPCK-M activities. Before measuring enzyme activity, the enzyme solution of the mitochondrial fraction was completely frozen at –30°C and thawed three times, followed by sonication for 30 sec using a sonication homogenizer.

The proteins in the enzyme solution were measured using a modification of Bradford's method (1976) to enable calculating the enzyme activity per unit protein.

G6Pase activity

The G6Pase activity was measured as described by Baginski *et al*. (1974). This assay is based on the measurement of inorganic phosphate released from the substrate glucose-6-phosphate by the enzyme G6Pase. In brief, 10 µL of enzyme solution was incubated at 37°C for 10 min, followed by the addition of 40 µL of 0.1 M sodium cacodylate buffer (pH 6.5):0.25 M sucrose/1 mM EDTA:0.1 M glucose-6-phosphate (5:1:2), and reacted at 37°C for 15 min. The reaction was stopped by mixing 100 µL of 2% ascorbic acid/10% trichloroacetic acid solution, centrifuged (1,000 ×*g*, 4°C, 10 min), and phosphoric acid was measured from the supernatant using the Phospha C Test kit (Wako) as recommended by the manufacturer.

FBPase activity

The FBPase activity was measured by modifying the methods described by Opie and Newsholme (1967) and Latzko and Gibbs (1974). In brief, 1.1 mL of 50 mM Tris-HCl buffer (pH 7.5; 6 mM $MgSO_4$-$7H_2O$, 1 mM EDTA-4Na, and 20 mM mercaptoethanol), 0.08 mL of 0.2 mM NADP, 0.08 mL of 3.5 U/mL phosphoglucose isomerase (Nacalai Tesque, Kyoto, Japan), 0.08 mL of 0.07 U/mL glucose-6-phosphate dehydrogenase (TOYOBO), and 0.03 mL of enzyme solution were mixed and incubated at 35°C for 5 min. The reaction was initiated by mixing 0.03 mL of 0.1 mM fructose-1,6-bisphosphate (Sigma) and the change in absorbance at 340 nm during the reaction was measured at 10-sec intervals for 5 min with an auto spectrophotometer (BioSpectrometer Kinetic, Eppendorf AG, Hamburg, Germany) at 35°C.

PEPCK-C and PEPCK-M activities

PEPCK activity was measured using a modified version of the method described by Walker *et al*. (1999). Final concentrations of 100 mM HEPES-KOH (pH 6.8), 100 mM KCl, 0.14 mM NADH, 25 mM dithiothreitol, 6 mM $MnCl_2$, 2 mM phosphoenolpyruvate, 1 mM ADP, 90 mM $KHCO_3$, and 6 U/mL malate dehydrogenase (TOYOBO) were mixed to make a reaction solution. After incubating the enzyme solution at 30°C for 3 min, the reaction was initiated by mixing 30 µL of the enzyme solution with 1 mL of the reaction solution, and the change in absorbance at 340 nm was measured at 10-sec intervals for 2 min with the auto spectrophotometer at 30°C.

PC activity

The PC activity was measured using a modified version of the method described by Utter and Keech (1963). A mixture of 1.22 mL of 0.1 M Tris-HCl buffer (pH 7.8; 3 mM potassium pyruvate, 16 mM $KHCO_3$, and 5 mM $MgCl_2$), 0.04 mL of 1 mM ATP, 0.04 mL of 0.05 mM acetyl-CoA, 0.04 mL of 0.17 mM NADH, and 0.02 mL of 21.9 U/mL malate dehydrogenase was incubated for 2 min at 30°C. The reaction was initiated by adding 20 µL of enzyme solution and the change in absorbance at 340 nm during the reaction was measured at 5-sec intervals for 2 min with the auto spectrophotometer at 30°C.

Calculation of enzyme activity

Enzyme activity was calculated using the following equations and data were converted to be expressed as activity per unit protein (mg) in the tissue.

FDPase, PC, and PEPCK activities:

activity (nmol/min/mg protein) = $V/(\varepsilon \times d \times v) \times (\Delta E/\Delta t) \times 1000 \times 1/P$,

where V is the final volume of the reaction, v is the volume of the enzyme solution, ε is the molar absorption coefficient (6.22 ×

10^3 for NADH), d is the path length (1 cm), $\Delta E/\Delta t$ is the rate of change in absorbance per minute, and P is the protein concentration of the enzyme solution (mg/mL).

G6Pase activity:

Activity (nmol/min/mg protein) = phosphate concentration (nmol/mL) × 1/t × 1/P,

where t is the enzyme reaction time (15 min) and P is the protein concentration of the enzyme solution (mg/mL).

Statistical analysis

Data are expressed as mean ± standard error. Comparisons between groups were performed using the Tukey–Kramer multiple-comparison test. Statistical significance between means was judged at $p < 0.05$.

Results

Fig. 1 shows the plasma glucose levels, yolk sac membrane weight, glucose content, and glycogen content of chick embryos. The plasma glucose level was 55.2 mg/dL on E8, but gradually increased and exceeded 100 mg/dL on E15; it then remained stable but resumed increasing on E21. The maximum value was 203.6 mg/dL at E21 and remained almost the same after hatching (Fig. 1A). The yolk sac membrane weight (wet weight without yolk) was 0.40 g on E3 when the sampling was started, but increased as incubation proceeded, reaching a maximum of 3.26 g on E15. After E17, the yolk sac membrane contracted toward hatching and reached a minimum (0.41 g) at C3, the last day of sampling. The yolk sac membrane was almost completely absorbed in the intestinal tract at C3 (Fig. 1B). The total amount of glucose contained in the yolk sac membrane was 0.39 mg at E4 and increased gradually throughout the incubation period, reaching a maximum at E17 (11.25 mg). After E17, the amount of glucose decreased as the wet weight of the yolk sac decreased, reaching 0.47 mg on C3 (Fig. 1C). The total amount of glycogen contained in the yolk sac membrane was 0.46 mg on E4 and thereafter tended to increase, similar to the trend for glucose. The maximum value was 102.51 mg glycogen on E17 (approximately 220 times higher than that on E4) and decreased after E17 to 4.39 mg on C3 (Fig. 1D).

Fig. 2 shows the changes in mRNA expression levels of key enzymes involved in glycogenesis (G6Pase, FBPase, PEPCK-C, PEPCK-M, and PC). The G6Pase mRNA expression levels remained similar between E3 and E20, with no significant differences. However, these mRNA levels increased significantly from E21 to hatching, reaching a maximum at C2. The FBPase mRNA levels increased significantly from E3 to E6, with the highest expression levels detected at E6 throughout the incubation period. Thereafter, FBPase mRNA levels tended to decrease gradually toward hatching, with the lowest expression level detected at C3. The PEPCK-C mRNA expression level increased from E3 to E7, reaching a maximum at E7. It then decreased significantly at E8 and remained at a constant level until E14. After E15, it increased again, peaked at E17, and then continued to decrease thereafter until C3. PEPCK-M mRNA expression levels both increased and decreased between E3 and E11, but tended to consistently increase thereafter. After reaching a maximum at E16, the PEPCK-M mRNA level tended to continuously decrease until E21 and remained at low levels after hatching. PC mRNA expression increased from E3 to E5 and reached a maximum at E5. After E5, it continued to decrease until E9 and thereafter maintained a low expression level until C3.

Conventional PCR experiment showed that the insulin-independent glucose transporter GLUT2 was expressed in the yolk sac membrane on all days between E3 and C3 (data not shown).

Fig. 3 shows the changes in enzyme activities. G6Pase activity increased from E3 to E9, peaking at E9. After E9, the G6Pase activity tended to decrease and showed a similar level of change until E18. After E18, it increased again and reached its maximum value at C3. FBPase activity (data only available from E6 due to the small amount of tissue) was the highest on E6, but decreased on E7, and thereafter tended to increase gradually until E17. After peaking at E17, the FBPase activity decreased significantly and remained at low levels until C3. PEPCK-C activity increased significantly between E3 and E5, reaching a maximum at E5; it decreased from E5 to E7, moderately increased between E7 and E10, decreased again, and remained low between E14 and E18. PEPCK-C activity tended to increase slightly from E18 to hatching and was maintained at a similar level thereafter. PEPCK-M activity remained low during the early incubation period and reached its maximum at E17. After E17, PECK-M activity maintained relatively stable. Throughout the incubation period, the enzymatic activity of PEPCK-M was lower than that of PEPCK-C. PC activity increased significantly from E3 to E5, reaching a maximum at E5. After a significant decrease from E5 to E6, the PC activity level remained low until C2 and then increased again to C3.

Fig. 4 shows the changes in lactate concentration in the yolk sac membrane. On E3, the first day of quantification, the lactate concentration was 0.53 mg/dL, but increased gradually toward E5, reaching a maximum value of 3.10 mg/dL. After E5, the lactate concentration tended to decrease until E12 (0.07 mg/dL on E12). Lactate tended to increase gradually before hatching with a concentration of 1.27 mg/dL at E21. After hatching, these values tended to decrease. Lactate concentrations in the plasma were also measured in the third week, which remained constant from E15 to E17, started to increase from E18, peaked with a significant increase at E19, and decreased significantly at E20 and E21 ($p < 0.05$ by Tukey–Kramer test, n = 7–9; data not shown).

Fig. 5 shows the changes in NEFA and triglyceride contents in the yolk sac membrane. The NEFA content was 1.34×10^{-4} mEq on E3 and continued to increase until E14. The maximum value was 1.59×10^{-2} mEq on E14 and tended to decrease after E15. The triglyceride content was 0.44 mg at E3 and continued to increase until E16. The maximum value was 27.42 mg at E16 and tended to decrease thereafter.

Discussion

The biochemical data obtained in the present study suggest that oxygen availability to the chick embryo during incubation is

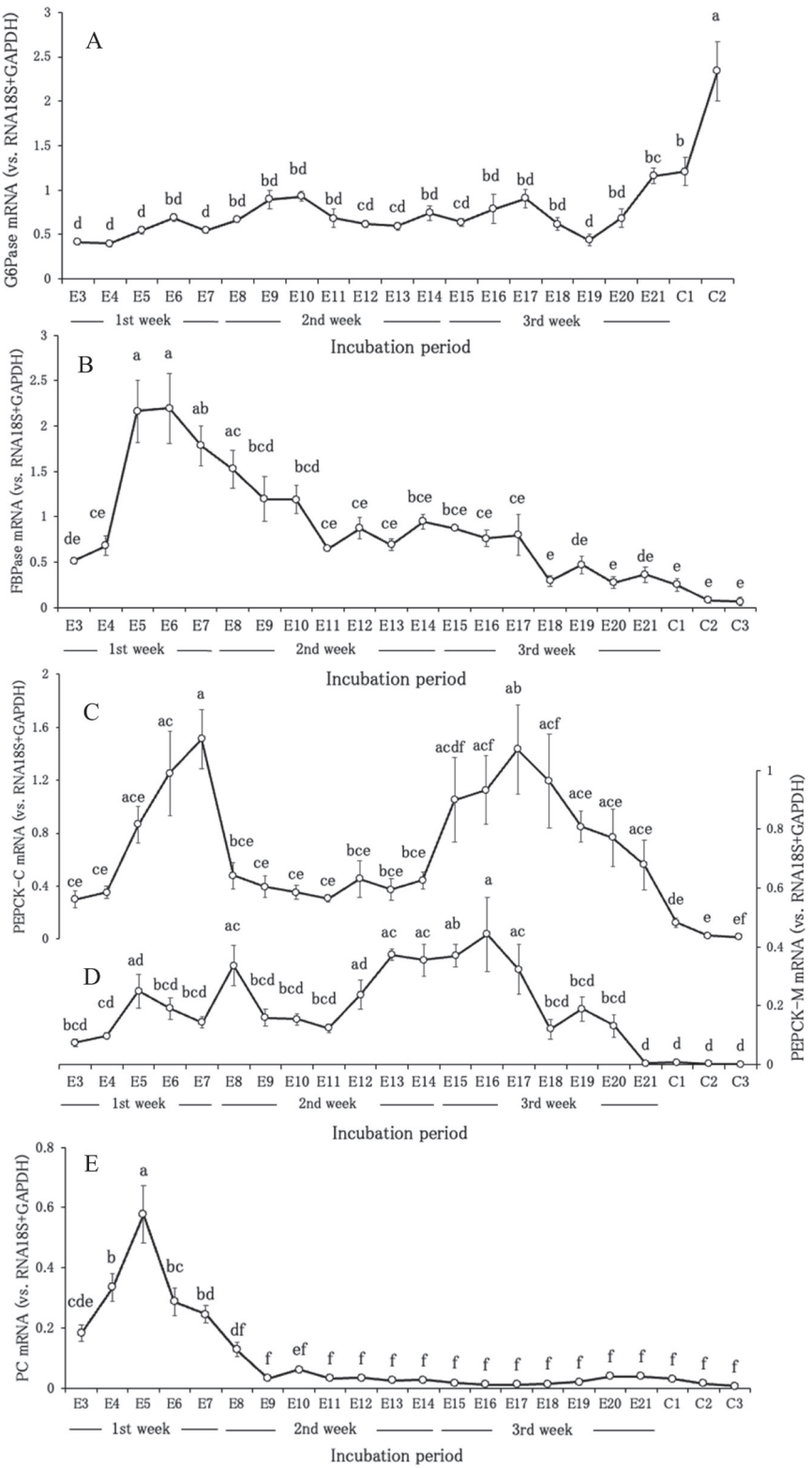

Fig. 2. **Changes in mRNA expression levels of key gluconeogenic enzymes in the yolk sac membrane during embryonic development (relative quantities expressed in arbitrary units; mean ± SEM, n = 7–9 per stage): (A) glucose-6-phosphatase (G6Pase), (B) fructose-1,6-bisphosphatase (FBPase), (C) phosphoenolpyruvate carboxykinase cytosolic isozyme (PEPCK-C), (D) phosphoenolpyruvate carboxykinase mitochondrial isozyme (PEPCK-M), and (E) pyruvate carboxylase (PC)**. Means without a common letter are significantly different within the same enzyme ($p < 0.05$, Tukey–Kramer multiple-comparison test).

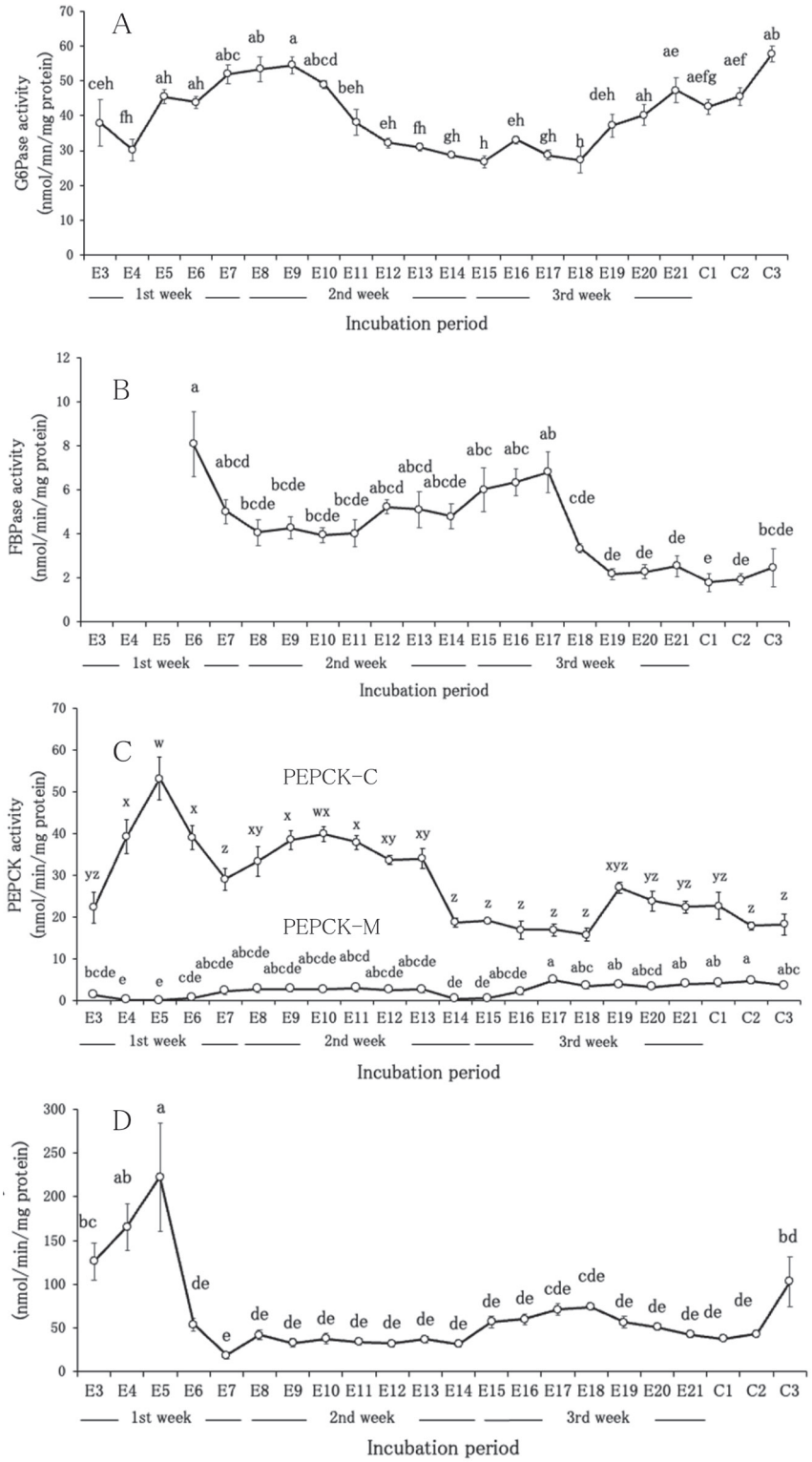

Fig. 3. **Changes in activities of key gluconeogenic enzymes in the yolk sac membrane during embryonic development (mean ± SEM, n = 7–9 per stage): (A) glucose-6-phosphatase (G6Pase), (B) fructose-1,6-bisphosphatase (FBPase), (C) phosphoenolpyruvate carboxykinase cytosolic isozyme (PEPCK-C), phosphoenolpyruvate carboxykinase mitochondrial isozyme (PEPCK-M), and (D) pyruvate carboxylase (PC).** Means without a common letter are significantly different within the same enzyme ($p < 0.05$, Tukey–Kramer multiple-comparison test).

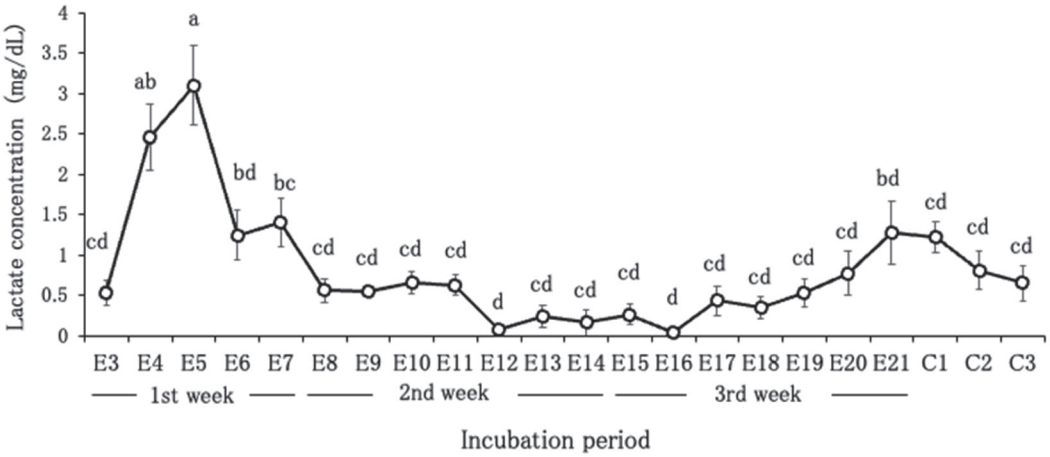

Fig. 4. **Lactate concentration in the yolk sac membrane during embryonic development of the chicken (mean ± SEM; n = 7–9).** Means without a common letter are significantly different ($p < 0.05$, Tukey–Kramer multiple-comparison test).

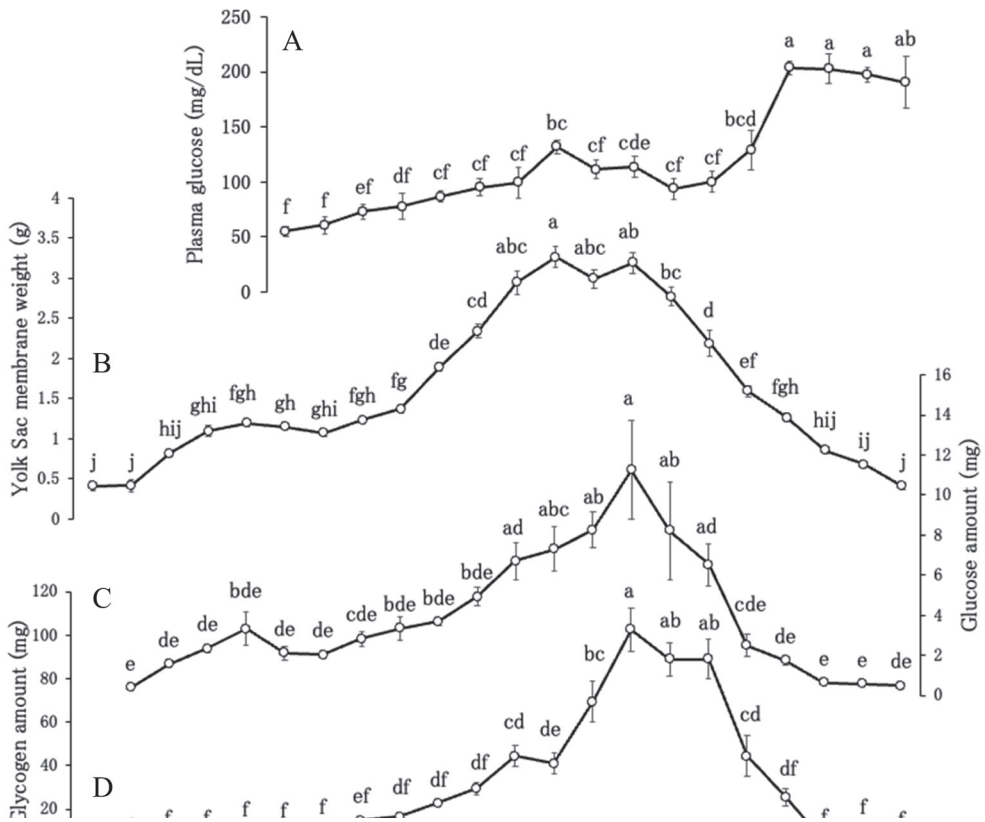

Fig. 5. **Levels of (A) non-esterified fatty acids (NEFAs) and (B) triglycerides in the yolk sac membrane during embryonic development of the chicken (mean ± SEM, n = 7–9 per stage).** Means without a common letter are significantly different within the same measurement category ($p < 0.05$, Tukey–Kramer multiple-comparison test).

highly variable. It is well established that during intensive exercise in animals, including humans (Spriet *et al*., 2000; Rovira *et al*., 2007; Kitaoka *et al*., 2014), energy production in the mitochondria (via the citric acid cycle and subsequent oxidative phosphorylation) becomes insufficient under an insufficient oxygen supply to the cells, and lactate generally accumulates in the skeletal muscle tissue. The changes in lactate concentration observed in the yolk sac membrane implied that a similar oxygen shortage occurred in these tissues, particularly during the first week of incubation. There was also a tendency for the lactate concentration to increase in the late third week, although the difference was not statistically significant.

First week to mid-second week of incubation

During incubation, the chorioallantoic membrane develops at approximately E5, reaching 75% of the inner eggshell membrane by approximately E9, and lines can be detected throughout the egg in contact with the inner eggshell membrane by approximately E12 (Romanoff, 1960a; Tazawa, 1980; Vleck and Bucher, 1998). Capillaries develop in the chorioallantoic membrane, allowing efficient gas exchange between the embryo and the surrounding atmosphere through the air cells and pores of the eggshell. In addition, an increase in oxygen transport is facilitated by an increase in the formation of red blood cells and a rise in hemoglobin levels (Tazawa, 1980). To the best of our knowledge, no previous study has directly demonstrated that the amount of oxygen supplied is lower than the demands of the embryo until the chorioallantoic capillaries fully develop. However, it has been argued (Vleck and Bucher, 1998; Moran, 2007; De Oliveira *et al*., 2008) that the embryo has poor access to the external air and is therefore in an oxygen-deficient state, as it breathes even in the very early stages of incubation (Kučera *et al*., 1984) when the chorioallantoic capillary is still developing. Accordingly, our results demonstrating high yolk sac membrane lactate levels, especially in the first week of incubation, support the assumption of oxygen deficiency in the early stages of incubation.

The Cori cycle occurs in mammals and birds (e.g., humans: Hoffer, 1990; chickens: Watford, 1985). In this cycle, lactate produced in the muscle by anaerobic contraction, for example, is taken up via the blood into the liver and converted into glucose by gluconeogenesis; the glucose is then secreted into the blood by the liver, taken up by the muscle in turn, and used to produce lactate.

In the Cori cycle, pyruvate is produced from lactate by lactate dehydrogenase, and three key unidirectional enzymes (enzymes involved in the regulatory steps of a metabolic pathway) of gluconeogenesis produce glucose (more precisely, glucose 6-phosphate) (Rui, 2014; Hatting *et al*., 2018). PC catalyzes the conversion of pyruvate to oxaloacetate and PEPCK converts oxaloacetate to phosphoenolpyruvate. After several bidirectional enzymatic reactions, FBPase unidirectionally converts fructose 1,6-bisphosphate into fructose 6-phosphate. Fructose 6-phosphate and glucose 6-phosphate are interconverted isomers. We found that both the gene expression levels and activities of these key enzymes (PC, PEPCK-C, and FBPase) were high in the yolk sac during the first week of incubation. These results suggest that the increase in lactate in the yolk sac membrane (or embryonic organs connected through blood vessels) could be used for gluconeogenesis in the yolk sac membrane. If the increased lactate in the yolk sac membrane is directly used for glycogenesis in this tissue, it could be considered an "intra-organ Cori cycle," which functions as a lactate-recycling system.

PEPCK isoenzymes

Similar to mammals, two PEPCK isoenzymes with different subcellular localizations are known in birds: mitochondrial (PEPCK-M) and cytosolic (PEPCK-C) types (Hod *et al*., 1986). In most mammals except rodents, both types of PEPCK are present in the liver and kidneys. Conversely, in adult chickens, only PEPCK-M is present in the liver and both types are present in the kidney (Shen and Mistry, 1979a, 1979b; Watford *et al*., 1981). Transient PEPCK-C expression has also been observed in the livers of chick embryos (Savon *et al*., 1993; Roy *et al*., 2013).

Yadgary and Uni (2012) reported the expression of PEPCK-C in yolk sac membranes from E11 to E21. However, the present study is the first to demonstrate the presence of both isoenzymes in the yolk sac membrane based on gene expression and enzyme activity results. Further studies on the different roles of these two isoenzymes in this tissue are required. However, as the enzyme activity of PEPCK-C was much higher (320-fold on E4, 3.5-fold on E17, and 31-fold on average during the experimental period; Fig. 3) than that of PEPCK-M, we expect that PEPCK-C is the isoenzyme that mainly contributes to gluconeogenesis in the yolk sac membrane.

When lactate is the main carbon source for gluconeogenesis, it is more effective for cells to use PEPCK-M, which does not require the transfer of reducing equivalents (NADH) associated with the reaction between the mitochondria and cytoplasm (Watford *et al*., 1981; Hod *et al*., 1986). However, cells can also use PEPCK-C for gluconeogenesis from lactate (via aspartate transaminase or malate dehydrogenase reactions) if this isoenzyme is available (Watford, 1985). In mammals, it has been suggested that PEPCK-M is not regulated by the factors known to regulate PEPCK-C, such as circulating hormones (Hanson and Reshef, 1997), and that PEPCK-M is instead constitutively expressed; indeed, PEPCK-M activity levels do not necessarily parallel changes in gene expression (Stark and Kibbey, 2014). Among the enzymes examined in the present study, the lack of a particular match between PEPCK-M mRNA and enzyme activity is consistent with these previous observations in mammals. The similar profile of changes in lactate concentration of the yolk sac membrane and PEPCK-C activity during incubation may suggest that gluconeogenesis from increased lactate is not only the steady state induced by PEPCK-M but is also regulated through the induction of PEPCK-C. Elucidating the mechanisms linking the increase in lactate in the yolk sac membrane and the induction of PEPCK-C expression and activity is a promising research topic. For example, the transcriptional regulation of PEPCK-C gene expression in response to intracellular pH has been observed in the mammalian kidney cell line LLC-PK1 (Curthoys

and Gstraunthaler, 2001). Thus, the chicken yolk sac membrane and liver provide an excellent model for studying the cellular regulation of gluconeogenesis.

Mid-second week to mid-third week of incubation

Lactate concentrations in the yolk sac membrane tended to be lower at the later stages of incubation, although not significantly different, from those on days other than E4 and E5 (the peak in the first week). These data suggest that aerobic metabolism, which does not produce a substantial amount of lactate, occurs from the mid-second to mid-third weeks of incubation. Both the gene expression and enzyme activity of PC were significantly lower than those detected during the first week, suggesting that gluconeogenesis from lactate is less active in later stages.

Bauer *et al*. (2013) used quantitative PCR, immunohistochemistry, and *in situ* hybridization techniques to show that yolk lipids are engulfed by the endodermal epithelial cells of the yolk sac membrane by endocytosis, processed by lysosomal enzymes, and packed into new lipoproteins (low-density and very-low-density lipoproteins) containing triglycerides, which are then secreted into the vasculature. We found that the triglyceride content of the yolk sac membrane increased markedly and was significantly higher at E16 than at earlier stages. These results are consistent with those of Yadgary *et al*. (2014), who analyzed the transcriptome of the yolk sac membrane and identified genes responsible for lipid metabolism and transport.

Sato *et al*. (2006) reported that the respiratory quotient (i.e., the ratio of carbon dioxide emissions to oxygen consumption) of E14 and E18 embryos in layers and broilers was approximately 0.7. Their results indicated that the energy source of the embryo at this stage is primarily lipids. Accordingly, lipids, including triglycerides, delivered from the yolk to the embryo via the yolk sac membrane and blood vessels are likely to play an important role as an embryonic energy source. In addition, we found that the NEFA content in the yolk sac membrane increased significantly from the mid-second week to the mid-third week of incubation. Triglyceride lipase activity has been observed in the yolk sac membrane at E11–E21 (Yadgary *et al*., 2013). Within the yolk sac membrane, increased triglyceride levels could thus be hydrolyzed by lipase, leading to increased NEFAs. The resulting NEFAs can be transported to the embryo as an energy source or reconstituted into new triglycerides.

Glycerol is produced when a lipase hydrolyzes triglycerides to yield NEFAs. Although glycerol can be metabolized to pyruvate and used as an energy source, it can also be used as a source for gluconeogenesis, either directly or via pyruvate. For example, Wang *et al*. (2020) conducted *in vivo* tracer experiments using ^{13}C labeling and found that adult mice fasted for 6–18 h used glycerol, but not lactate, as a substantial source of gluconeogenesis (whether glycerol was directly converted to glucose or through conversion to pyruvate).

Gluconeogenesis from glycerol requires FBPase catalysis. In the present study, while the gene expression of FBPase tended to decrease gradually until the mid-third week, the enzyme activity remained high throughout incubation. This suggests that gluconeogenesis from glycerol is possible. We speculate that during this period, glycerol from the breakdown of increased triglycerides is the primary source of gluconeogenesis in the yolk sac membrane.

Late third week of incubation

During the late third week (E18), the lactate concentration in the yolk sac membrane tended to increase. Lactate concentration in the plasma increased significantly at E19. The observed increase in lactate concentration may reflect an increased anaerobic environment in the embryo. At the end of the incubation period, the oxygen supply from outside the eggshell is considered to be insufficient to meet the embryo's metabolic needs (Vleck and Bucher, 1998). Tazawa *et al*. (1983) noted a decrease in blood oxygen pressure in chick embryos as development progressed toward the end of incubation.

In contrast, enzyme activities during the late third week did not change significantly, except for an increase in G6Pase activity. PEPCK-C activity tended to be higher, but the difference was not statistically significant. Gene expression levels also tended to remain unchanged or decreased, except for a significant increase in G6Pase expression. Therefore, we considered the glycogenic activity in the yolk sac membrane to be low.

During this period (E18 onward), the weight of the yolk sac membrane and the amounts of glucose and glycogen gradually and significantly decreased. The conversion of glycogen stored in the yolk sac membrane to glucose and its delivery to the embryo may be responsible for the decrease in these quantities, leading to an overall decrease in yolk sac membrane weight. The glucose delivered to the embryo is a source of energy for embryonic movement and external respiration (pipping) during hatching, as proposed by De Oliveira *et al*. (2008).

Storage and secretion of glucose: the yolk sac membrane

Glucose 6-phosphate, produced by gluconeogenesis in the yolk sac membrane, is metabolized through the glycolytic pathway (as in the Cori cycle), stored as glycogen, or released into the blood as glucose. The last option should result in simply increasing blood glucose levels, using the glucose as energy for development and hatching, or sending it to embryonic organs such as the liver for storage as glycogen.

The glycogen and glucose contents of the yolk sac membrane increased toward the E17 peak. This trend agrees with previous reports (Thommes and Just, 1964; Yadgary and Uni, 2012); Yadgary and Uni (2012) reported that the gene expression of glycogen synthase in the yolk sac membrane gradually, almost linearly, increased during E11–E21. These results indicated that glycogen was continuously stored in the yolk sac membrane throughout the incubation period. Willier (1968) examined the detailed histology of the subcellular localization of glycogen in the yolk sac membrane of E5–E15 chicken embryos, and suggested the synthesis, storage, and transport of glycogen from the apical (yolk) to the basolateral (vascular) side of the yolk sac epithelium.

G6Pase activity significantly increased or tended to increase until E9 and after E18, but the activity was maintained throughout the incubation period. These data are in agreement with Ku-

suhara and Ishida's (1974) enzyme histochemical observations of the yolk sac epithelium, in which G6Pase activity was strongly positive and uniformly detected in the cytoplasm from E12 to E20. The presence of G6Pase allows the glucose in the yolk sac membrane to be secreted into the blood, because glucose, but not glucose 6-phosphate, can pass through glucose transporters in the plasma membrane. The findings of Kusuhara and Ishida (1974) and those of our study indicate that the chicken yolk sac is an organ that secretes glucose into the blood, similar to the functions of the liver and kidneys.

The gene expression of GLUT2 in the yolk sac membrane was observed from E3 to C3, the result in line with the previous report by Nakazawa *et al*. (2011) for E2–E4. Yadgary *et al*. (2011, 2014) observed the expression of GLUT2 and another glucose transporter, the sodium glucose transporter (SGLT1), between E11 and E21. In the mammalian cells of the small intestine (Drozdowski and Thomson, 2006) and kidneys (Ghezzi *et al*., 2018), SGLT1 is localized apically and GLUT2 is localized basolaterally. Although the histological localization of these transporters in the cells of the yolk sac membrane has not been reported, the daily presence of GLUT2 from E3 to C3 found in this study suggests that glucose synthesized and stored in the yolk sac epithelium is secreted into the blood vessels during this period, which may increase blood glucose levels.

Dynamics of blood glucose in the chicken embryo

Birds are characterized by a state of hyperglycemia and their blood glucose levels increase progressively during embryonic development to reach this state. Previous studies investigating blood glucose dynamics in chicken embryos during incubation have shown that blood glucose levels tend to increase toward hatching. Interestingly, this increase does not appear to be linear. For example, Roy *et al*. (2013) reported that blood glucose levels did not increase significantly within E9–E13, E14–E17, and E18–E21. In the present study, after a significant increase in blood glucose levels at E15, they increased significantly again only at E21. The differences in blood glucose profiles between studies may be due to the different chicken breeds used. However, the fact that there was no increase found between specific periods can be assumed to mean that during that period, the newly generated glucose was mainly used for a process other than increasing blood glucose levels. In line with the results of the present study, it can be assumed that glucose is mainly converted to glycogen for storage. This was supported by a significant and sustained increase in glycogen in the yolk sac membrane between E15 and E17.

In the present study, using embryos of laying hens, the metabolites, gene expression, and enzyme activity were measured daily in a single experimental run from as early as possible during the incubation period to immediately after hatching. This experiment allowed us to present a coherent picture of glucose production by the yolk sac membrane, most likely in response to environmental (e.g., oxygen supply) and developmental needs during the incubation period. These results provide a milestone for future detailed studies on individual processes. Furthermore, this study raises several critical biochemical issues that remain unsolved concerning the yolk sac membrane, which Willier (1968) called the "way station" of the transfer of yolk material between the yolk and the chick embryo, including what (hormones, metabolic factors, intracellular signaling molecules) regulates the point-switching, when the switching occurs, and the size of the respective metabolic traffic. The metabolic contribution of the embryonic liver gradually increases during the last half of the incubation period as the organ weight gradually increases after approximately E12. Thus, it will be fascinating to reveal the inter-organic circulation and exchange of metabolites between the liver, kidney, and yolk sac membrane required to proceed with successful embryonic development and hatching, and these experiments are currently underway by our research group. Possible differences between sexes and commercial strains of embryos should also be thoroughly examined.

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Author Contributions

M.S. and A.I. designed and conducted the experiments, analyzed the results, and wrote the manuscript. M.Y. designed the study, discussed the results, and prepared the manuscript. All authors contributed to the experimental purpose, methods, results analysis, and discussions.

Conflicts of Interest

The authors declare there is no conflict of interest.

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